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Borsig, Lubor ; Vlodavsky, Israel ; Ishai-Michaeli, Rivka ; Torri, Giangiacomo ; Vismara, Elena

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## Sulfated Hexasaccharides Attenuate Metastasis by Inhibition of P-selectin and Heparanase<sup>1</sup>

Lubor Borsig\*, Israel Vlodavsky<sup>†</sup>,  
Rivka Ishai-Michaeli<sup>‡</sup>, Giangiacomo Torri<sup>§</sup>  
and Elena Vismara<sup>¶</sup>

\*Zürich Center for Integrative Human Physiology, Institute of Physiology, University of Zürich, Zürich, Switzerland;

<sup>†</sup>Cancer and Vascular Biology Research Center, Bruce Rappaport Faculty of Medicine, Technion, Haifa, Israel;

<sup>‡</sup>Sharett Institute, Hadassah-Hebrew University Medical Center, Jerusalem, Israel; <sup>§</sup>Istituto di Ricerche Chimiche e Biochimiche "G. Ronzoni" via Giuseppe Colombo, Milan, Italy; <sup>¶</sup>Dipartimento di Chimica, Materiali e Ing. Chimica "G. Natta" Politecnico, Via Mancinelli, Milan, Italy

### Abstract

Development of compounds that target both heparanase and selectins is emerging as a promising approach for cancer therapy. Selectins are vascular cell adhesion molecules that mediate tumor cell interactions with platelets, leukocytes, and the vascular endothelium. Heparanase is an endoglycosidase that degrades heparan sulfate in the tumor microenvironment, cell surfaces, and vessel wall. Acting together, these molecules facilitate tumor cell arrest, extravasation, and metastasis. Here, we report the preparation of novel semisynthetic sulfated tri mannose C-C-linked dimers (STMCs) endowed with heparanase and selectin inhibitory activity. The P-selectin specificity of the STMC was defined by the anomeric linkage of the C-C bond. This STMC hexasaccharide is an effective inhibitor of P-selectin *in vivo*. We show that selective inhibition of heparanase attenuates metastasis in B16-BL6 melanoma cells, expressing high levels of this endoglycosidase, but has no effect on the metastasis of MC-38 carcinoma cells that express little or no heparanase activity. P-selectin-specific STMC attenuated metastasis in both animal models, indicating that inhibition of tumor cell interaction with the vascular endothelium is critical for cancer dissemination. Thus, the small size, the stability of the C-C bond, and the chemically defined structure of the newly generated STMCs make them superior to heparin derivatives and signify STMCs as valuable candidates for further evaluation.

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### Introduction

The control of cancer metastasis remains the major obstacle in treatment of cancer patients. Metastasis is a multiple-step process encompassing tumor cell release from primary sites, their survival in the circulation, and extravasation in distant tissues [1]. Metastasis is determined by the cellular origin, intrinsic features of the tumor, and the adhesive properties of tumor cells.

Heparanase, the only mammalian endoglycosidase that cleaves heparan sulfate (HS), is upregulated in essentially all human tumors examined [2–7]. A causal involvement of heparanase in tumor metastasis was demonstrated by increased lung, liver, and bone colonization of cancer cells after overexpression of the heparanase gene and by a marked decrease in metastatic potential of cells subjected to

Abbreviations: STMC, sulfated tri mannose C-C-linked dimer

Address all correspondence to: Lubor Borsig, PhD, Institute of Physiology University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.

E-mail: lborsig@access.uzh.ch

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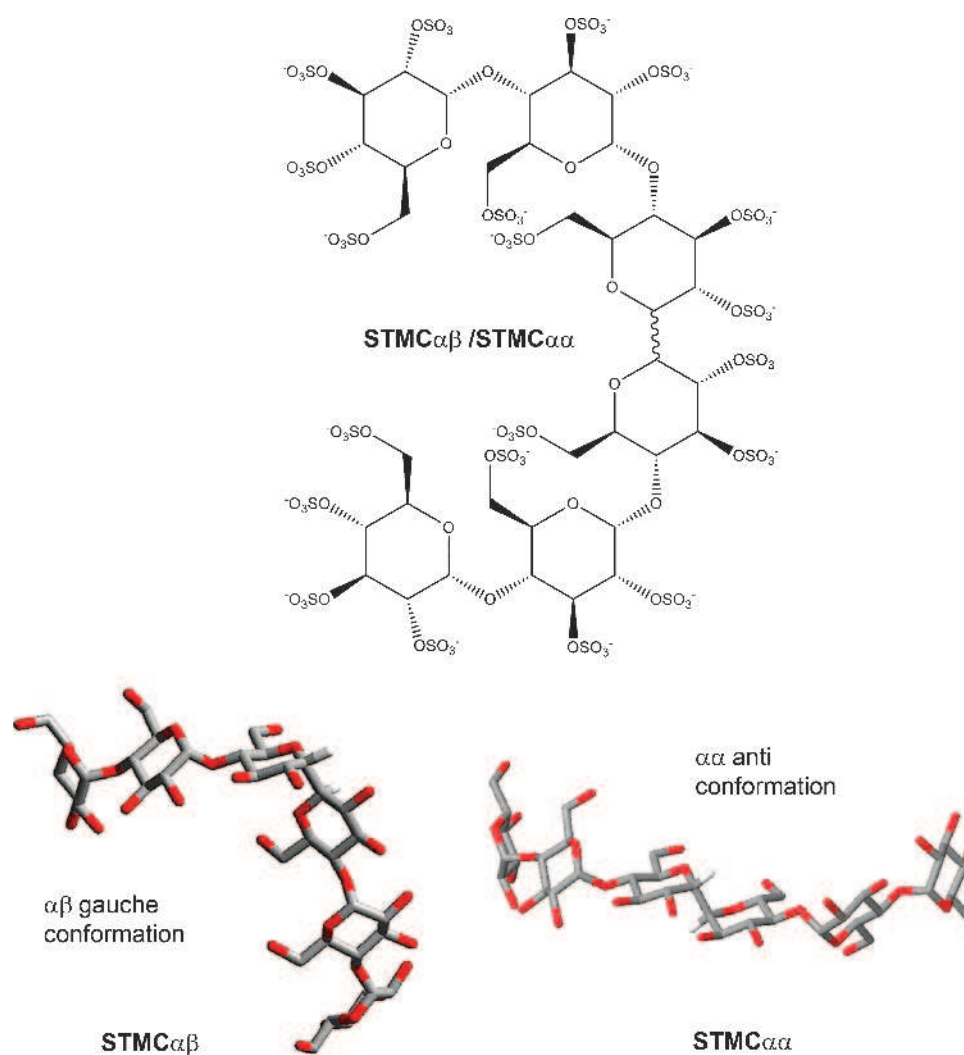
heparanase gene silencing [8,9]. Moreover, inhibition of heparanase resulted in attenuation of metastasis in a number of animal models [8–12]. A significant role of heparanase in tumor angiogenesis and lymphangiogenesis was demonstrated, applying similar experimental approaches [2,7,13]. Clinically, increased heparanase levels are most often associated with increased tumor metastasis, high microvessel density, and reduced patients' survival time after operation [2–4,6,14,15].

In another set of studies, the adhesive properties of tumor cells to vascular cell adhesion molecules, selectins, have been shown to mediate tumor cell interaction with platelets, endothelium, and leukocytes. Inhibition of selectin-mediated tumor cell interaction with blood constituents resulted in attenuation of tumor metastasis in a number of animal models [16–20].

Several laboratories, including ours, are developing heparin-mimicking compounds that compete with HS and thereby inhibit heparanase and selectin prometastatic activities [10,21–23]. Heparin has long been known to possess antiheparanase activity [24] and to effectively inhibit P- and L-selectins [25,26]. Results from several clinical trials using unfractionated heparin and low-molecular-weight heparin (LMWH) in preventing thromboembolic complication in advanced stage cancer patients indicated that heparin prolonged

survival [27] probably owing to a direct effect on the tumor, potentially through inhibition of heparanase enzymatic activity [28]. However, the use of heparin or LMWH as anticancer agents is limited because of the risk of inducing adverse bleeding complications. Moreover, heparin exhibits a number of biologic activities including inhibition of thrombin generation [29], release of tissue factor pathway inhibitor from endothelial surfaces [30], modulation of growth factors' receptor binding and activity [31], affecting angiogenesis [32], heparanase enzymatic activity [33], and selectin-mediated cell interactions [34,35]. Despite a significant progress in the analysis of heparin activities affecting cancer progression, there is a need for a synthetic small molecule inhibitor of heparanase and/or selectins to be tested as a potential antimetastatic treatment.

In the present study, we tested novel semisynthetic hexasaccharide compounds for their capacity to inhibit heparanase and/or P-selectin activities *in vitro* as well as experimental metastasis *in vivo*. For this purpose, we have synthesized hexasaccharide mimics of maltohexaose sulfate in which a central glycosidic bond was substituted by a hydrolase-resistant C-C bond. Maltohexaose sulfate was chosen because of its bioequivalence with phosphomannopentaose sulfate (PI-88), a potent carbohydrate-based heparanase inhibitor currently



**Figure 1.** Structures and preferred conformation of STMC $\alpha,\alpha$  and STMC $\alpha,\beta$ .

being subjected to phase 2/3 clinical trials in cancer patients [36]. The sugar chains of these compounds are characterized by the presence of an interglycosidic C-C bond expected to confer chemical and metabolic stability compared with malto-oligosaccharides.

## Materials and Methods

### Preparation of Compounds

Halo-sugars' electroreduction on silver cathode has been used to prepare double sugar units cancelled through the formation of stable interglycosidic C-C bonds. This procedure is accompanied by the loss of a halide anion from an electrochemically reduced halo-sugar, and the radical reactivity dictates the statistic distribution of products with formation of  $\alpha,\alpha$ :  $\alpha,\beta$ :  $\beta,\beta$  C-C bonds (1:2:1) [37,38]. Tri-maltose C-C-linked dimers TMC $\alpha,\alpha$  and TMC $\alpha,\beta$  were prepared by acetobromomaltotriose electrochemical reduction followed by deacetylation [39]. Sulfation of TMC $\alpha,\alpha$  and TMC $\alpha,\beta$  was performed by addition of 590 mg of sulfur trioxide pyridine complex (3.68 mmol, 10 Eq/Eq -OH) to a pyridine solution of TMC (0.0185 mmol,  $4 \times 10^{-3}$  M), resulting in STMC $\alpha,\alpha$  and STMC $\alpha,\beta$ , respectively (Figure 1). The reaction mixture was warmed to 80°C and stirred for 6 hours, avoiding moisture with a CaCl<sub>2</sub> trap. The mixture was cooled down to room temperature, neutralized with a saturated solution of NaHCO<sub>3</sub>, and exhaustively evaporated under vacuum. The solid residue was dissolved in water and separated on TSK column. STMC $\alpha,\alpha$  and STMC $\alpha,\beta$  were fully characterized by NMR analysis (E. Vismara et al., unpublished data).

### Cell Lines and Reagents

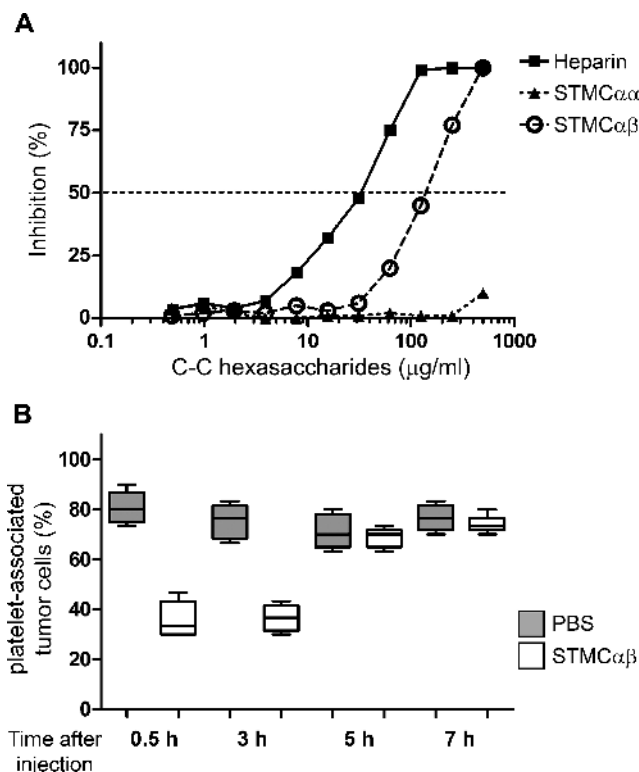
Mouse colon carcinoma cell line MC-38 stably expressing GFP, MC-38GFP [17], and mouse melanoma cell line B16-BL6 [8] were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). Human colon carcinoma cells LS180 (ATCC, Manassas, VA) were grown in  $\alpha$ -minimum essential medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). All reagents were from Sigma (St. Louis, MO) unless otherwise stated.

### Mice

Wild-type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Zürich Center for Integrative Human Physiology.

### Inhibition of P-selectin

Ability of STMCs to inhibit adhesion of LS180 cells to immobilized P-selectin was examined as described previously [34]. Briefly, an ELISA plate (Nunc, Rochester, NY), coated overnight with soluble Protein A, was blocked with 1% bovine serum albumin in Hank's balanced salt solution for 30 minutes at room temperature and incubated with mouse P-selectin chimera (400 ng/well) for 3 hours at room temperature. Calcein AM-labeled LS180 tumor cells were added to the plate in the presence or absence of serially diluted STMCs at concentrations ranging from 0.58 to 500  $\mu$ g/ml. After 1 hour of incubation while rotating at 4°C, adherent cells were quantified by measuring the fluorescence with a GENios ELISA reader (Tecan,



**Figure 2.** (A) Selectin inhibitory activity of C-C hexasaccharides. The ability of STMC $\alpha,\alpha$  and STMC $\alpha,\beta$  hexasaccharides to inhibit P-selectin-mediated adhesion of LS-180 human colon carcinoma cells was compared to that of unfractionated heparin as described in Materials and Methods. Data are representative of three independent experiments. (B) Bioavailability of STMC $\alpha,\beta$  *in vivo*. Platelet adhesion to intravenously injected MC-38GFP cells was analyzed in mice receiving STMC $\alpha,\beta$  and compared to PBS-injected mice. The number of tumor cells positive for platelet staining was determined in lungs from mice killed at 30 minutes, 3 hours, or 7 hours after injection as described in Materials and Methods. The difference in platelet/tumor cell association between control-injected mice (PBS) and STMC $\alpha,\beta$ -injected mice was found to be statistically significant at 30 minutes and 3 hours by one-way ANOVA ( $P < .001$ ).

Männedorf, Switzerland). The half-maximal inhibitory concentration (IC<sub>50</sub>) values were calculated from three independent experiments.

### Coagulation Assays

Automatic determination of partial thromboplastin time (PTT) of samples was performed in duplicates by mixing pooled normal plasma with PTT reagent (FSL actin; Dade Behring, Deerfield, IL). After 10 minutes, the clotting time was determined on a Sysmex CA-1500 analyzer (Diamond Diagnostics, Holliston, MA). Photometric determination of anti-Xa activity was performed to evaluate the activity of STMCs in human plasma on Sysmex CA-1500 analyzer, using the LMWH Kit (Chromogenics, Mölndal, Sweden).

### Heparanase Activity Assay

Preparation of sulfate-labeled extracellular matrix (ECM)-coated dishes and determination of heparanase enzymatic activity were performed as described in detail elsewhere [40,41]. STMCs were tested for their ability to inhibit heparanase, as previously described [33].

Briefly, sulfate-labeled ECM coating the surface of 35-mm culture dishes was incubated (4 hours, 37°C, pH 6.0) with constitutively active (GS3) [42] recombinant human heparanase (120 ng/ml) in the absence or presence of 5 µg/ml of each STMCs. The incubation medium containing sulfate-labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS, and their radioactivity was counted in a  $\beta$ -scintillation counter. Degradation fragments of HS side chains were eluted at  $0.5 < K_{av} < 0.8$  (peak II, fractions 15–35). Nearly intact heparan sulfate proteoglycan (HSP) was eluted just after the  $V_0$  ( $K_{av} < 0.2$ , peak I, fractions 3–12). We have previously demonstrated that labeled fragments eluted in peak II are degradation products of HS because they were 1) fivefold to sixfold smaller than intact HS side chains, 2) resistant to further digestion with papain and chondroitinase ABC, and 3) susceptible to deamination by nitrous acid [40].

### Platelet–Tumor Cell Aggregation In Vivo

C57BL/J6 mice were killed at various time points after intravenous injection of tumor cells, and frozen lungs sections were incubated with anti-CD41 antibody (Becton Dickinson, Mountain View, CA), followed by detection with goat antirat antibody conjugated with Alexa568 (Invitrogen) as described [34]. The extent of platelet–tumor

cell association was quantified in 20 view fields (40 $\times$  magnifications) by immunofluorescence microscopy.

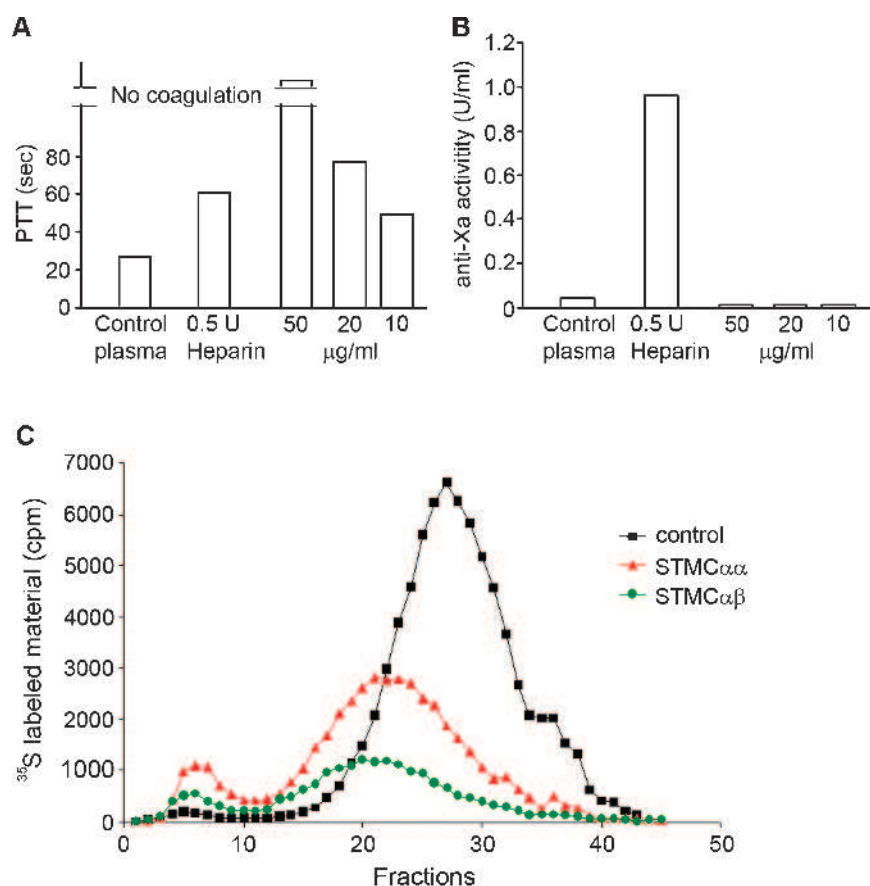
### Experimental Metastasis

C57BL/J6 wild-type mice were intravenously injected with 300,000 MC-38GFP cells or B16-BL6 melanoma cells [43]. Some mice received 150 µg of STMCs 10 minutes before tumor cell injection. Mice injected with B16-BL6 melanoma cells were killed after 14 days, and the number of lung metastatic foci was counted. Mice injected with MC-38GFP cells were killed after 28 days and macroscopically evaluated. Metastatic burden in the lungs was determined by measurements of GFP in the lung homogenate [17].

## Results

### Sulfated Hexasaccharides as Potential Inhibitors of Metastasis

Modified heparins that block P-selectin and/or heparanase have been tested as potential inhibitors of metastasis [10,33]. We aimed at synthesizing small oligosaccharide structures endowed with inhibitory activity of P-selectin and/or heparanase and testing their therapeutic potential in mouse models of metastasis.



**Figure 3.** Heparanase and anticoagulant activity of STMCs. (A and B) Anticoagulant activity. STMCs were tested for effect on PTT (A) and anti-Xa activity (B) as described in Materials and Methods. Shown are the results obtained with STMC $\alpha\beta$ . Same results were obtained with STMC $\alpha\alpha$ . Data are representative of three independent determinations, and the variation did not exceed  $\pm 10\%$  of the mean. (C) Heparanase inhibitory activity. The ability of STMC $\alpha\alpha$  and STMC $\alpha\beta$  hexasaccharides to inhibit recombinant heparanase enzymatic activity was determined as described in Materials and Methods. Data are representative of three independent experiments.



Halo-monosaccharides electroreduction on silver cathode allows dimerization of a carbon-centered radical and affords C-disaccharide mimics [38]. By extending this technique to oligosaccharides, we succeeded in preparing sulfated tri-maltose C-C-linked dimers STMC $\alpha,\alpha$  and STMC $\alpha,\beta$  (Figure 1). A detailed description of the synthetic procedure is provided elsewhere [38].

### STMCs Inhibit P-selectin Binding in a Linkage-Specific Manner

Attenuation of metastasis in the absence of P-selectin or its inhibition by native and chemically modified heparins has been documented in a number of animal models [10,16,34,44]. To investigate C-C-linked hexasaccharides as potential inhibitors of P-selectin, we tested the ability of both STMC $\alpha,\alpha$  and STMC $\alpha,\beta$  to inhibit adhesion of LS-180 colon carcinoma cells to immobilized P-selectin [10]. STMC $\alpha,\beta$  showed a good inhibitory activity of P-selectin (IC<sub>50</sub> 150  $\mu$ g/ml), although less effective than unfractionated heparin (IC<sub>50</sub> 40  $\mu$ g/ml) (Figure 2A). In contrast, STMC $\alpha,\alpha$  exerted no P-selectin inhibitory activity, indicating that change of the C-C linkage from  $\alpha\alpha$  to  $\alpha\beta$  is critical for binding to P-selectin.

To determine an effective dose of STMC $\alpha,\beta$  for the metastasis experiments, we first analyzed its bioavailability *in vivo*. Tumor cells carrying selectin ligands are known to form tumor cell emboli mediated primarily through platelet P-selectin [16,34]. We intravenously injected 150  $\mu$ g of STMC $\alpha,\beta$  10 minutes before injection of MC-38GFP mouse colon carcinoma cells. Mice were killed at different time points between 30 minutes and 7 hours after injection, and the extent of platelet-tumor cell association was quantified (Figure 2B). Approximately 80% of tumor cells in the lung tissue of PBS-injected mice were associated with platelets at all times. Injection of STMC $\alpha,\beta$  reduced platelet aggregation on tumor cells to approximately 40% during the first 3 hours, but there was no inhibition at later time points (5 and 7 hours). These results indicate that STMC $\alpha,\beta$  is biologically active for at least 3 hours after tumor injection and is relatively rapidly cleared from the circulation.

### Anticoagulant Activity of STMCs

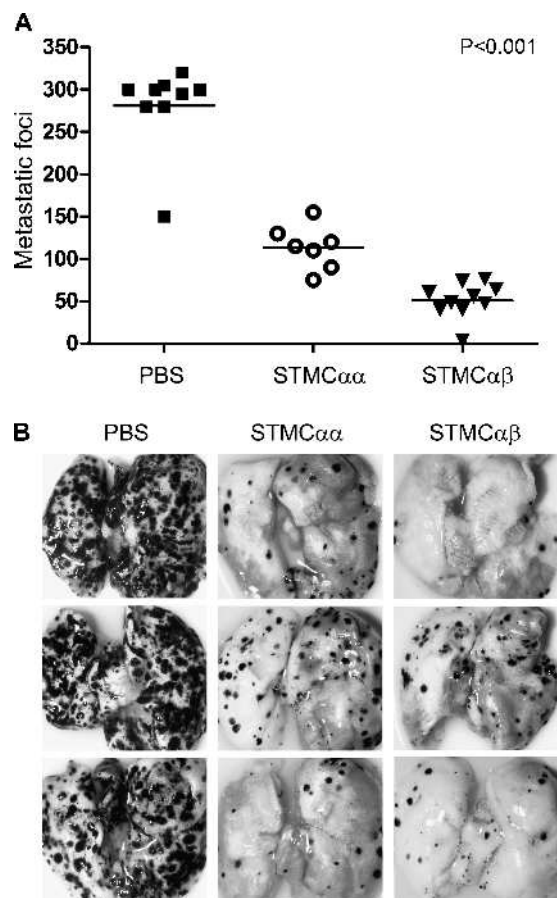
Both compounds STMC $\alpha,\alpha$  and STMC $\alpha,\beta$  exhibited a high, dose dependent anticoagulant activity (PTT, at 10  $\mu$ g/ml comparable to that of 0.5 U heparin), but were devoid of anti-Xa activity (Figure 3, A and B).

### STMCs Effectively Inhibit Heparanase Enzymatic Activity

Heparanase activity is associated with cancer progression in a variety of cancers and its inhibition by heparin derivatives attenuates tumor growth and metastasis [2–5,9,12]. Using a naturally produced sulfate-labeled ECM as a substrate [40,41], we tested the ability of both STMC $\alpha,\alpha$  and STMC $\alpha,\beta$  to inhibit heparanase enzymatic activity. As demonstrated in Figure 3C, compound STMC $\alpha,\beta$  was more effective than compound STMC $\alpha,\alpha$ , yielding 85% and 58% inhibition by 5  $\mu$ g/ml, respectively.

### STMCs Attenuate Metastasis Primarily by Inhibition of P-selectin

To determine the ability of STMCs to attenuate metastasis, we intravenously injected wild-type mice with 150  $\mu$ g/mouse of STMC followed by injection of B16-BL6 melanoma cells 10 minutes later. B16-BL6 cells were shown to express P-selectin ligands as well as sig-

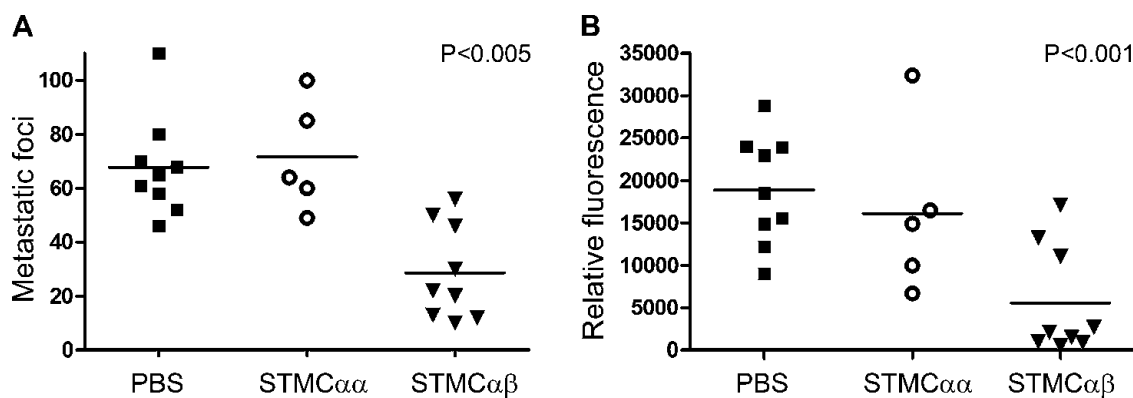


**Figure 4.** C-C hexasaccharides attenuate metastasis of B16-BL6 melanoma cells. (A) Mice were intravenously injected with 150  $\mu$ g of STMC $\alpha,\alpha$  or STMC $\alpha,\beta$  10 minutes before injection of  $3 \times 10^5$  B16-BL6 cells. Fourteen days after injection mice were killed, and the lungs were dissected and evaluated for the number of metastatic foci/lung. Statistical significance was determined by one-way ANOVA ( $P < .001$ ). (B) Representative images of lungs derived from mice injected with STMC $\alpha,\alpha$  or STMC $\alpha,\beta$  versus control (PBS).

nificant amounts of heparanase [10]. Heparanase-specific STMC $\alpha,\alpha$  reduced metastasis by ~65%, whereas the P-selectin-specific STMC $\alpha,\beta$ , which inhibits both P-selectin and heparanase, was more effective, yielding 82% inhibition (Figure 4). Next, we tested both STMCs in the MC-38GFP colon carcinoma model [10,17]. Intravenous injection of STMCs was followed by injection of MC-38GFP cells, and mice were killed after 28 days (Figure 5). The P-selectin-specific STMC $\alpha,\beta$  attenuated metastasis to similar levels as observed with P-selectin-deficient mice [10,17], whereas STMC $\alpha,\alpha$  had no effect, in agreement with the little or no heparanase activity observed in MC-38 colon carcinoma cells [10]. Taken together, these findings indicate that the newly synthesized hexasaccharides effectively attenuated experimental metastasis by targeting P-selectin and that inhibition of heparanase enzymatic activity is a valid approach in heparanase-expressing tumor cells.

### Discussion

Several studies have shown that the antimetastatic activity of heparin is based on its ability to inhibit heparanase and selectins [23,33,34,45]. The bioactive moieties of natural polysaccharides reside in specific or



**Figure 5.** Experimental metastasis of MC-38 colon carcinoma cells is efficiently attenuated by STMC $\alpha,\beta$  endowed with selectin inhibitory activity. C57BL/6 mice were intravenously injected with 150  $\mu$ g of STMC $\alpha,\beta$  10 minutes before injection of  $3 \times 10^5$  MC-38GFP cells and killed 28 days later. (A) The number of metastatic foci/lung representing initial metastatic seeding of tumor cells was counted. (B) The extent of lung colonization was quantified by measurement of GFP fluorescence in lung homogenates. Statistical difference was found only for the STMC $\alpha,\beta$  compound as determined by *t* test ( $P < .001$ ).

unspecific oligosaccharides mostly consisting of more than four carbohydrate units [46]. Owing to their small molecular size, oligosaccharides are expected to have enhanced bioavailability than their polymeric precursors. These considerations led to isolation of sequences from natural oligosaccharides and development of glycan mimetics having the capacity to inhibit heparanase or selectins [47–49]. Sulfated maltohexaose has been identified to be an efficient inhibitor of tumor growth and metastasis, primarily because of inhibition of heparanase [47]. PI-88, a mixture of highly sulfated oligosaccharides, reached phase 3 clinical development for hepatocellular carcinoma. A new class of heparanase-inhibiting HS mimetics based on anomerically pure, fully sulfated oligosaccharides was recently optimized for anticancer drug development [49]. In the case of selectin inhibitors, the early developments of sialyl Lewis X–based glycan mimetics has been replaced by development of nonglycoside small molecule inhibitors [50]. In the present work, we tested semisynthetic sulfated malto-oligosaccharides for their potential to inhibit heparanase and P-selectin. These glycan mimetics are characterized by the presence of an interglycosidic C–C bond, which, similar to other C-glycosides that are less vulnerable to metabolic processing than their O-analogs, is expected to confer improved chemical and metabolic stability relative to malto-oligosaccharides [51]. Molecular modeling and conformational analysis have shown that the interglycosidic C–C bond modifies the geometry of the sugar chains, increasing their conformation rigidity [39]. Whereas STMC $\alpha,\beta$  was found to be an effective inhibitor of P-selectin, the conformational change to STMC $\alpha,\alpha$  completely eliminated its selectin binding activity (Figure 2A), emphasizing the high specificity of this interaction. In contrast, both STMCs inhibited heparanase enzymatic activity, the  $\alpha\beta$  configuration being more effective (Figure 3C), indicating a less restricted specificity for interaction with heparanase compared with selectins. Because the conformational flexibility of oligosaccharides is critical for their binding to proteins and, consequently, for their bioactivity [52], our observations provide evidence that a rigid C–C structures defines the specificity of selectin binding.

STMCs effectively attenuated metastasis in both the B16 melanoma and MC-38 colon carcinoma systems (Figures 4 and 5). Because the heparanase-specific STMC $\alpha,\alpha$  did not affect metastasis of MC-38GFP carcinoma cells expressing no heparanase (Figure 5),

tumor-derived heparanase, as opposed to heparanase contributed by other blood-borne cells (i.e., neutrophils, platelets), seems to be critical for metastasis in this experimental setting [10]. STMC $\alpha,\beta$  that efficiently inhibits P-selectin was a better inhibitor of lung colonization by B16-BL6 melanoma and MC-38GFP carcinoma cells than STMC $\alpha,\alpha$  (Figure 5). Likewise, modified heparins endowed with P-selectin–inhibitory activity effectively attenuated metastasis, to an extent similar to that observed in P-selectin–deficient mice [10,22,23,53]. The rapid cell surface expression of P-selectin on platelets and endothelial cells on activation makes P-selectin one of the earliest molecules mediating cell adhesion [54,55]. Accumulating evidence indicates that P-selectin–mediated interactions contribute to cancer progression (reviewed in Laubli and Borsig [55,56] and Ludwig et al. [57]). Yet, the recently developed specific selectin inhibitors are being tested only in different inflammatory situations such as ischemia-reperfusion injury, atherosclerosis, and deep vein thrombosis [50,58]. Here, we provide evidence that sulfated mannose–based hexasaccharides (STMC $\alpha,\beta$ ) specifically inhibit P-selectin and thereby attenuate experimental metastasis (Figure 5). Thus, STMC $\alpha,\beta$  represents one of the smallest glycan-based selectin inhibitor that is active *in vivo*.

High heparanase expression by multiple myeloma cells is associated with enhanced bone metastasis [6] and modified non-anticoagulant glycol-split heparins endowed with heparanase inhibitory activity have been shown to effectively attenuate myeloma tumor growth and bone metastasis [10,28]. Similarly, inhibition of heparanase either by modified heparins [10] or by the newly developed STMCs hexasaccharides (Figure 4) attenuated metastasis, further confirming the critical involvement of heparanase in metastasis. Importantly, the heparin-derived compounds effectively inhibited xenograft tumor growth and spontaneous metastasis of human myeloma [9,28] and sarcoma [59]. Synthesis of STMCs is being scaled up to enable their evaluation in the same systems.

Non-anticoagulant heparin–derived inhibitors of metastasis have been developed and tested in a number of laboratories (reviewed in Casu et al. [60], Borsig [61], and Kragh and Loechel [62]). However, heparin-based inhibitors exhibit a limited bioavailability and poor pharmacokinetics *in vivo*. Unlike heparin, the synthetic hexasaccharides (STMCs) presented in this work represent stable, small size

single entity oligosaccharides, expected to be more readily optimized for drug development in terms of target (e.g., selectin *vs* heparanase) specificity and bioavailability. Notably, the therapeutic potential of compounds targeting heparanase and/or selectins is not restricted to cancer, taking into account their involvement in inflammatory diseases [63–65] and renal dysfunction [66].

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